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Structural basis of allosteric regulation of eukaryotic phosphofructokinases

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The enzyme 6-phosphofructokinase (Pfk) catalyses the formation of fructose 1,6-bisphosphate from fructose 6-phosphate and MgATP and contributes to the control of glycolysis in prokaryotic and eukaryotic cells. The catalytic activity is tightly regulated in a wide variety of organisms by diverse positive (e.g. fructose-2,6-bisphosphate, AMP) and negative (e.g. ATP, citrate) effectors.

Eukaryotic Pfk has evolved by a process of tandem gene duplication and fusion to yield a protein with a much more complex structural organization and allosteric regulation. The N-terminal half of a Pfk subunit obviously retained the catalytic function, whereas in the C-terminal half allosteric ligand binding sites have evolved from former catalytic and regulatory sites.

Pichia pastoris Pfk (PpPfk) is with ~1 MDa the most complex and probably largest eukaryotic Pfk [2]. We have determined the crystal structure of full-length *Pp*Pfk to 3.05 Å resolution in the T-state [1]. *Pp*Pfk forms an $(\alpha\beta\gamma)_4$ dodecamer of D_2 symmetry with dimensions of 161 x 157 x 233 Å mainly via interactions of the α-chains. The N-terminal domains of the α- and β-chains have folds that are distantly related to glyoxalase I, but the active sites are no longer functional. Interestingly, these domains located at the two distal ends of this protein along the long two-fold axis form a $(\alpha\beta)_2$ dimer as does the core Pfk domains, however, the domains are swapped across the tetramerization interface. In *Pp*Pfk, the unique γ-subunit participates in oligomerization of the αβ-chains. This modulator protein was acquired from an ancient SAM-dependent methyltransferase. The identification of novel ATP binding sites, which do not correspond to the bacterial catalytic or effector binding sites, point at marked structural and functional differences between bacterial and eukaryotic Pfk.

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Molecular mechanism of metal selective uptake by neutrophil-activating protein

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Helicobacter pylori is a pathogen associated with gastric diseases, including gastric ulcers and stomach cancers. *Helicobacter pylori* neutrophil-activating protein (HP-NAP) is reported to be a major 17 kDa antigen of *H. pylori*. This protein promotes the neutrophil adhesion to endothelial cells and the production of reactive oxygen species which is thought to be a cause of cancer. HP-NAP is a ferritin-like iron storage protein and forms a hollow spherical dodecamer whereas ferritin forms 24mer. Crystal structure of HP-NAP was solved in 2002 at 2.5 Å resolution[1]. The structure contained a Fe atom per subunit at a ferroxidase center. We previously determined the metal-free structure at 2.2 Å resolution[2]. The crystal shows higher symmetry (space group: *F*432, Zanotti's one: $P2_1$) and a high water content of 65.9% (*V*s). Therefore, the *F*432 crystal is worthwhile for soaking experiments. In comparison with Zanotti's structure, the large conformational changes by the metal coordination were observed for three amino-acid residues (His37, Asp52 and Glu56). In order to estimate the selective mechanism of iron uptake, storage and elimination, we have determined the crystal structures containing various metal ions.

HP-NAP was expressed as a His-tag fused protein and crystallized using the hanging drop vapor diffusion method at 20 °C. To prevent the protein from aggregation, 0.1 M L-arginine was added during purification and crystallization processes. Crystals were grown against 25% ethylene glycol precipitant. By cocrystallization method, crystals including Fe^{2+} or Cd^{2+} ions were obtained using the reservoir solution containing each metal ion. Soaking methods were also performed for Fe^{2+} and Zn^{2+} ions. Anomalous difference data were collected at the Photon Factory in Japan. Ferrous ions incorporated into HP-NAP are probably oxidized to ferric ions instantly by HP-NAP's ferroxidase activity. When Fe^{2+} is added to the droplet, the crystal color is changed to be tinged with yellow. Therefore, Fe atoms in the structure were assigned as ferric ions and refined.

Cd^{2+} and Fe^{3+} structures through cocrystallization were obtained at 2.2 and 2.7 **Å** resolution, respectively. Each subunit contains fourteen cadmium ions and two ferric ions, respectively, and these ions were chelated in a different manner. Fe^{3+} and Zn^{2+} structures obtained through soaking method, were refined at 3.0 and 2.5 **Å** resolution, respectively. Three ferric ions and six Zn^{2+} ions were chelated in ordinary mononucleated manners. Number of metal ions through soaking method is greater than that through cocrystallization. Negative-charged residues are abundant in the inner surface of HP-NAP, which contributes to this additional iron storage mechanism. The most major metal ions were located at the ferroxidase center of the subunit interface. At these metal chelation sites, metal ions adopt the different coordination involved in one or two water ligands. Zn^{2+} and Cd^{2+} ions form binuclear coordination and Fe^{3+} builds mononucleated chelation.

There are two different kinds of pore channels, one of which must be the selective metal ion path from the outer hydrophilic shell to the inner space and vice versa, and are formed at the 3-fold rotational symmetry axes. In Fe^{3+} and Zn^{2+} structures, metal ions are clearly observed at one of the pores.

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Crystal structure of the allosteric-defective chaperonin $GroEL_{E434K}$ mutant

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The chaperonin GroEL adopts a double-ring structure with various modes of allosteric communication. The simultaneous positive intra-ring and negative inter-ring co-operativities alternate the functionality of the folding cavities in both protein rings. Mutation of glutamic acid 434 to lysine, a residue located at the rings interface, alters the negative inter-ring co-operativity [1]. The crystal structure of the mutant chaperonin $GroEL_{E434K}$ has been determined at low-resolution (4.5 Å) and compared with two other structures: the wild-type GroEL [2] and the allosteric-defective $GroEL_{E461K}$ [3] mutant. Despite the allosteric-defective behavior of $GroEL_{E434K}$, its structure remains strikingly similar to that of the wild type GroEL.

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Structure determination of toxin complex from *Clostridium botulinum* serotype D

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Clostridium botulinum produces seven immunologically distinct serotypes of neurotoxin (BoNT; 150 kDa), classified as A to G. BoNT forms a large toxin complex (TC) varying from 300 kDa up to 900 kDa, through association with non-toxic non-hemagglutinin (NTNHA; 130 kDa) and three kinds of hemagglutinin (HA) subcomponents, HA-70, HA-33 and HA-17 (70, 33 and 17 kDa, respectively). Recently, we proposed a hypothetical 14-mer subunit model of the botulinum serotype D TC on the basis of the transmission electron microscopy (TEM) observations, X-ray crystallography and biochemical analyses [1]. TEM images revealed an ellipsoidal-shaped structure with three-arms attached. The crystal structure of HA-33/HA-17 complex was determined first by the molecular replacement (MR) method, and that of BoNT was generated by the homology modeling using serotypes A and B BoNTs as templates. Following our novel model, Nakamura *et al.* solved the trimer-like structure of HA-70 subcomponent [2]. Thereby, only the structure of NTNHA is remaining unknown at present. Crystals of the M-TC, a complex of BoNT and NTNHA, was obtained before [3]. The crystals diffracted to 8Å resolution and were not suitable for structure analysis. Unfortunately, no crystals could be obtained ever again.

In the present study, the crystal structure of the HA-33/HA-17 complex was determined again by the SIRAS method using a platinum derivative to confirm the structure resulted from the MR method. All diffraction data were collected on an in-house X-ray system; a Rigaku R-AXIS VII image plate detector equipped with a Rigaku FR-E+ SuperBright X-ray generator. The platinum derivative was obtained by soaking crystals in 10 mM K_2PtCl_4 solution containing 7% PEG8000, 0.1 M MES (pH 6.5) and 0.1 M $MgCl_2$ for 10 min at room temperature. Native and derivative data sets were collected to 2.15 and 2.30 Å resolution, respectively, at 93K. The final structure was refined to R_{cryst} of 20.3% and R_{free} of 24.7% with one heterotrimeric HA-33/HA-17 complex in the asymmetric unit. The SIRAS structure confirmed that the lower electron density of HA-17 is not due to the phasing bias originated from the MR method but due to its flexible structure.

Our ultimate goal is to conclude the unique subunit structure of botulinum TC by combining the various techniques. As one of new attempts, we are trying to elucidate the three-dimensional shape of the NTNHA protein or complexes including NTNHA and the arrangement of each subcomponents in the TC using biological small angle X-ray scattering (BioSAXS). In addition, the measurements of protein-protein interaction in solution by quartz crystal microbalance (QCM) technique are currently in progress.

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Structural insights into a dodecameric machine – The RuvBL1/RuvBL2 complex

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RuvBL1 (RuvB-like 1) and its homolog RuvBL2 are evolutionarily highly conserved AAA^+ ATPases essential for many cellular activities. They play an important role in chromatin remodeling, transcriptional regulation and DNA damage repair. RuvBL1 and RuvBL2 are overexpressed in different types of cancer and interact with major oncogenic factors, such as β-catenin and c-myc regulating their function. We solved the first three-dimensional crystal structure of the human RuvBL complex with a truncated domain II and show that this complex is competent for helicase activity. The structure reveals a dodecamer consisting of two heterohexameric rings with alternating RuvBL1 and RuvBL2 monomers bound to ADP/ATP, that interact with each other via the retained part of domain II. Interestingly, truncation of domain II led to a substantial increase in ATP consumption of RuvBL1, RuvBL2 and their complex. In addition, we present evidence that DNA unwinding of the human RuvBL proteins can be autoinhibited by domain II, which is not present in the homologous bacterial helicase RuvB. Our data give new insights into the molecular arrangement of RuvBL1[1] and RuvBL2 and strongly suggest that *in vivo* activities of these highly interesting therapeutic drug targets are regulated by cofactors inducing conformational changes via domain II in order to modulate the enzyme complex into its active state.

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